Immunization with cationized BSA inhibits progression of disease in ApoBec-1/LDL receptor deficient mice with manifest atherosclerosis

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Short title: cBSA immunization inhibits atherosclerosis progression

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Abbreviations: alum, aluminum salt-containing gel; apoB, apolipoprotein B; cBSA, cationized BSA; LDL, low density lipoprotein

Abstract

Immune responses against modified self-antigens generated by hypercholesterolemia play an important role in atherosclerosis identifying the immune system as a possible novel target for prevention and treatment of cardiovascular disease. It has recently been shown that these immune responses can be modulated by subcutaneous injection of adjuvant. In the present study we immunized 25-week old ApoBec-1/LDL receptor deficient mice with manifest atherosclerosis with adjuvant and two different concentrations of the carrier molecule cationized BSA (cBSA). Plasma levels of Th2-induced apolipoprotein B (apoB)/IgG1 immune complexes were increased in the cBSA immunized groups verifying induction of immunity against a self-antigen. Mice were sacrificed at 36 weeks of age and atherosclerosis was monitored by *en face* Oil red O staining of the aorta. Immunization with 100 µg cBSA inhibited plaque progression, whereas the lower dose (50 µg) did not. In addition, the higher dose induced a more stable plaque phenotype, indicated by a higher content of collagen and less macrophages and T cells in the plaques. Moreover, there was an increased ratio of $Foxp3^+/Foxp3^-$ T cells in the circulation suggesting activation of a regulatory T cell response. In conclusion, we show that immunization with cBSA induces an immune response against apoB as well as an activation of Treg cells. This was associated with development of a more stable plaque phenotype and reduced atherosclerosis progression.

Introduction

Accumulation and oxidation of low density lipoprotein (LDL) in the vascular wall is a key event in the development of atherosclerosis (Hansson et al. 2006). Oxidized LDL particles promote inflammation, leading to influx of mononuclear phagocytes. These cells ingest the oxidized lipoprotein particles and give rise to foam cells and contribute to the atherogenic process by orchestrating inflammatory responses (Galkina and Ley 2009). Oxidation of LDL results in a large variety of oxidized lipid-phospholipid-protein adducts. Modifications of lysines or histidines in these new molecules render them immunogenic (Palinski and Witztum 2000). Activation of Th1 immune responses against these modified self antigens have been shown to contribute to disease progression while immune responses leading to generation of antibodies and Treg activation appear protective (Nilsson and Hansson 2008).

We have recently shown that aluminium salt-containing gel (alum) injected into a hypercholesterolemic environment can absorb antigens from modified LDL and activate immune responses against these in a manner that contributes to protection against early atherosclerosis (Wigren et al. 2009). The alum immunization was shown to activate immune suppressive Treg cells and downregulate activation markers on $CD4^+$ T cells (Wigren et al. 2009). Alum is the most widely used adjuvant in clinical vaccines. Another experimental study has also demonstrated atheroptotective properties of alum immunization

(Khallou-Laschet et al. 2006). In addition to a general immune stimulatory mechanism of an adjuvant, alum has been shown to induce cell death and to thereby enhance antigen presentation by dendritic cells (Kono and Rock 2008; Kool et al. 2008). In the present study we aimed to extend our previous findings by including a carrier in the immunization procedure, to evaluate if the carrier could enhance an atheroprotective immune response in older ApoBec-1/LDL receptor deficient mice with already existing plaques.

The most commonly used carrier proteins are capable of imparting immunogenecity to covalently coupled haptens. Some of the more useful ones include keyhole limpet hemocyanin (KLH) and BSA because of their proven efficacies (Coligan et al. 2001). However, one disadvantage of KLH may be its large size that makes it more likely to precipitate during cross-linking, making the complex difficult to handle. Studies comparing native and cationized BSA (cBSA) showed an alteration of the immunogenic and regulatory properties due to the cationization (Muckerheide et al. 1987a; Muckerheide et al. 1987b). Both *in vivo* and *in vitro*, cBSA was highly immunogenic, producing significantly greater responses than the native BSA. C57BL/6 mice produced a significant antibody response to BSA when immunized with cBSA, in contrast to the immunization with native BSA (Muckerheide et al. 1987a; Muckerheide et al. 1987b). Taken together, this indicates that cBSA, because of its positive

charges, has greater affinity for the negatively charged cell surface membrane of antigen presenting cells resulting in a more efficient uptake of the antigen.

Here we tested the hypothesis that using an immunization strategy combining alum with a carrier would produce an atheroprotective response against a self antigen in mice with manifest advanced disease. In our previous study, it was implicated that the atheroprotective alum effect was based on activation of Treg cells. Thus, we decided to use several administrations of the carrier, a strategy known to induce immunosuppression.

Material and Methods

Animals and immunization protocol

We used female ApoBec-1/LDL receptor deficient mice on C57BL/6 background (Jackson Laboratories, Bar Harbor, Maine), which express full length apoB-100 in their LDL particles. The mice (n=10-21 per group) were fed a high fat diet (0.15% cholesterol, 21% fat, Lantmännen, Sweden) from 4 weeks of age, provided *ad libitum.* The diet was changed to chow diet 20 weeks later. Thereafter the mice were immunized at 25, 27, 29, 31, 33, 35 and 36 weeks of age with cBSA. Each injection contained 50 µg cBSA (cBSA low) or 100 µg cBSA (cBSA high) dissolved in 0.083 mol/L sodium phosphate 0.9 mol/L NaCl pH 7.2, according to the manufacture's protocol (77652, Pierce, Rockford, Ill, USA) and with alum (Pierce) as adjuvant. The mice were sacrificed 3 days after the last immunization by intraperitoneal injection of 300 µl Xylazine (Rompun, Bayer Health care), and Ketamine (Ketalar, Pfizer), 3:1:1 vol/vol/vol and exsanguinated by cardiac puncture, perfused with 5 ml PBS (pH 7.4) and 5 ml of the fixative Histochoice (Amresco, Solon, Ohio). The heart was collected and stored in Histochoice at 4°C until processing. The descending aorta was dissected free of connective tissue and fat, cut longitudinally, and mounted *en face* lumen-side up on ovalbumin (Sigma-Aldrich, St. Louis, MO) coated slides and stored in Histochoice. Plasma was collected from cardiac punction and stored at -80°C until assayed. The Local Animal Care and Use Committee at Lund University approved the experimental protocol used in the study.

Staining of the descending aorta

En face preparations of the descending aorta were washed in distilled water, dipped in 78% methanol, and stained for 40 minutes in 0.2% Oil-Red-O dissolved in 78% methanol/0.2 mol/L NaOH as previously described (Brånén et al. 2001). The cover slides were mounted with a water soluble mounting media L-550A (Histolab, Göteborg, Sweden). Oil Red O stains lipids red, which makes the plaques bordeaux colored. Stained area (bordeaux colored) and total aortic areas were quantified blinded by microscopy and computer aided morphometry (Olympus Micro Image, Hamburg, Germany and BioPix iQ 2.0, Göteborg, Sweden).

Analysis of plaque macrophage, CD3 and collagen content

The heart was embedded in OCT (Optimal Cutting Temperature; Tissue-Tek, Zoeterwoulde, The Netherlands) and frozen sections of 10 μm were collected. Modified Masson's trichrome staining using Buin´s solution (Histolab), Biebrich-Scarlet-Acid Fuchsin solution, Phosphomolybdic-Phosphtungstic acid solution (Sigma-Aldrich) and aniline blue (VWR International AB, Stockholm, Sweden) was used to assess plaque collagen content. Slides used for staining with rat anti-mouse MOMA-2 antibodies (monocyte/macrophage, BMA Biomedicals, Augst, Switzerland) diluted in 10% rat serum in PBS incubated at +4°C over night, were first fixed in ice-cold acetone for 10 minutes, washed in PBS for 5 minutes, incubated with Triton-X100 (Merck Chemicals, Darmstadt,

Germany) for 5 minutes and thereafter blocked with 10% mouse serum in PBS for 30 minutes and quickly dipped in PBS. Biotinylated rabbit anti-rat IgG (BA-4001, Vector Laboratories, Burlingame, CA) was used as secondary antibody and DAB detection kit for color development (Vector). For CD3 detection, slides were fixed in acetone, washed and permeabilized using Triton-X100 followed by incubation with 10% goat serum. Slides were incubated with rabbit anti-human CD3 (A0452, DakoCytomation, Fort Collins, CO) diluted in 2% goat serum at 4°C over night and thereafter incubated with biotinylated goat anti-rabbit IgG (BA-1000, Vector) and DAB. Omissions of the primary or secondary antibodies were used as controls. Stained area was quantified blinded by microscopy and computer aided morphometry (BioPix IQ 2.0, Göteborg, Sweden).

Cell preparation and flow cytometry

Two days before the mice were killed; blood was drawn from the saphenous vein into heparin coated tubes (Sarstedt, Landskrona, Sweden). Blood was centrifuged at 500*g* for 15 minutes at 4°C. Cells were incubated with a Fc-receptor blocking antibody (FcR;CD16/32; Biolegend, San Diego, CA) for 5 minutes followed by incubation with CD4-PE/Cy7 (GK1.5) and CD25-APC (PC61) diluted in cell staining buffer (all from Biolegend) for 30 minutes at 4°C. Erythrocytes were lyzed with red blood cell lysis buffer (Sigma-Aldrich) and remaining cells were washed with FC buffer (1% fetal calf serum (Invitrogen

Life Technologies, Carlsbad, CA) and 0.5 mM EDTA in PBS). The cells were resuspended in Fix/Perm solution (eBioscience, San Diego, CA), washed with permeabilization buffer (eBioscience) and blocked with FcR block prior to incubation with Foxp3-PB (FJK-16S, eBioscience) for 30 minutes at 4°C. Cells were washed with permeabilization buffer and resuspended in FC buffer. Measurements were performed using a Cyan ADP (Beckman Coulter, Fullerton, CA), analyses were performed using Summit (Beckman Coulter, version 4.3) and gating was adjusted using a negative control staining.

Proliferation assay

Splenocytes in single cell suspension were prepared by pressing spleens through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ). Erythrocytes were removed using red blood cell lysing buffer (Sigma). Cells were cultured in culture media (RPMI 1640 media containing 10% heat-inactivated fetal calf serum, 1 mmol/L sodium pyruvate, 10 mmol/L Hepes, 50 U penicillin, 50 μg/mL streptomycin, 0.05 mmol/L β-mercaptoethanol and 2 mmol/L L-glutamine, GIBCO, Invitrogen) in 96-well round bottom plates (Sarstedt, Landskrona, Sweden). In proliferation assays 2 x 10^5 splenocytes/well were cultured alone or with 2.5 μg/mL Concanavalin A (Con A, Sigma) for 90 hrs. To measure DNA synthesis the cells were pulsed with 1 μCi [methyl-^{3}H] thymidine (Amersham, Uppsala, Sweden) during the last 16 hrs. Macromolecular material was then harvested on glass fiber filters using a Printed Filtermat A (1450-421,

Wallac Oy, Turku, Finland). Filters were air-dried and the bound radioactivity was measured in a beta-counter (Wallac 1450 MicroBeta, Ramsey, MN).

Analysis of cytokines in cell supernatants

Cytokine concentrations in cell supernatants of ConA stimulated splenocytes were analyzed using a mouse Th1/Th2 9-Plex (IFN-γ, IL-1β, TNF-α, IL-2, IL-12, IL-4, IL-5, IL-10 and KC (keratinocyte chemoattractant)) Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, MO), following the instructions of the manufacturer.

ApoB immune complex ELISA

Microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) were coated with rabbit anti-apolipoprotein B antibody (ABcam, Cambridge, MA) according to manufacturers instructions. The plasma antibodies were detected using antibodies recognizing mouse IgM or IgG (1:5000, Jackson ImmunoResearch, West Grove, PA), IgG1 (1:2000) or IgG2a (1:500, both BD Pharmingen, San Jose, CA, IgG2a cross reacts to IgG2c).

Plasma cholesterol and triglyceride analysis

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, Infinity™ Cholesterol and Triglyceride (INT and Sigma, respectively).

Statistical analysis

Analysis of data was performed using unpaired *t* test or Mann Whitney test for skewed data. Data are presented as mean±standard deviation. Analysis was performed using GraphPad Prism 5.01 and a level of $P<0.05$ was considered significant.

Results

Increased levels of plasma apoB/IgG and IgG1 immune complexes in mice immunized with cBSA

In a previous study, young apoE deficient mice were immunized with alum alone, resulting in atheroprotection (Wigren et al. 2009). Alum was shown to precipitate oxidized LDL antigens at the injection site, with a consequential increase in plasma IgG1 recognizing an oxidized LDL epitope. However, in mice with manifest advanced disease it is more difficult to induce atheroprotection. Thus, in the present study we aimed to evaluate if the effect could be more pronounced by combining alum with a carrier, by testing two different concentrations (50 and 100 μg) of cBSA. Induction of an oxLDL-specific immune response was examined by determining plasma levels of immune complexes containing apoB-100, the major protein of the LDL particle. In mice immunized with cBSA, the levels of plasma apoB/IgG immune complexes (2.05±1.03 absorbance (abs) units in the high dose group and 2.34±0.55 abs units in the low dose group versus 0.26±0.18 abs units in non-treated mice, $P<0.0001$) and apoB/IgG1 immune complexes (1.53±1.16 abs units in the high dose group and 1.63±0.97 abs units in the low dose group versus 0.01±0.01 abs units in non-treated mice, $P<0.0001$) were higher in immunized mice versus non-treated mice (Figure 1). No differences were detected in the plasma levels of apoB/IgM and apoB/IgG2c immune complexes (data not shown). Thus, since the IgG subtype IgG1, is associated with Th2

immunity, the results indicate that the cBSA immunization induce a more anti-inflammatory immune response. No differences in antibody levels recognizing oxidized LDL was found (data not shown).

cBSA immunization induces reduction of atherosclerosis and a more stable plaque phenotype

To study the effect of the cBSA immunization on atherosclerosis development, *en face* preparations of the descending aorta were analyzed. The plaque area at 24 weeks of age (before the treatment started) was 5.3±2.1%. The progression of the plaque area was found to be significantly reduced in mice immunized with a 100 μg dose of cBSA compared to non-treated mice and mice immunized with a lower (50 μg) dose (13.4±5.5% versus 17.9±5.6 and 19.6±4.3, respectively; $P<0.05$) (Figure 2). The mice receiving a high dose of cBSA also had a more stable plaque phenotype, when plaque morphology was assessed by the content of macrophages (Figure 3A) and presence of collagen (Figure 3B). These mice were found to have significantly less macrophages (17.9±6.6% versus 31.9±10.8%, $P<0.001$ in non-treated mice and 25.5±9.9%, $P<0.05$ in the lower dose group) and a trend towards presence of more collagen (52.5±5.3% versus 46.6±11.3%, ns in non-treated and 48.7±7.3%, $P<0.07$ in the lower dose group) in the subvalvular lesions. Further, we calculated the collagen/macrophage ratio as an indicator of plaque stability (Figure 3C). The ratio was significantly increased in mice immunized with the higher dose of cBSA compared to non-

treated mice and mice immunized with a lower dose (2.97±0.81 versus 1.62±0.63, P=0.0014 and 2.17±0.84, P<0.05, respectively). Taken together, these findings indicate that immunization with cBSA can result in protection against atherosclerosis development.

Less $CD3^+$ T cells in aortic subvalvular lesions of mice immunized with a high dose of cBSA

Atherosclerotic plaques contain an increased amount of immune cells, as macrophages and T cells. Thus, we also determined the presence of $CD3^+$ T cells in the aortic plaques (Figure 4). We found less T cells in the subvalvular lesions, in accordance with less macrophages, in the group immunized with a higher dose of cBSA (4.09±2.26% versus 8.40±2.66%, P<0.001 in non-treated mice and 6.24±3.14%, P=0.06 in the lower dose group).

Mice immunized with a higher dose of cBSA have more Treg cells versus T effector cells

To further characterize the effect of the immunization we analysed the T cell population in the blood with flow cytometry. We have previously shown that immunization with alum alone significantly increased the presence of $CD4^+CD25^+Foxp3^+$ Treg cells in apoE deficient mice and was associated with a reduced development of atherosclerosis (Wigren et al. 2009). Here we demonstrate that cBSA has a dose-dependent effect on the T cell population

(Figure 5). The group immunized with a higher concentration of cBSA was found to have a dramatic decrease of activated $CD4^+CD25^+Foxp3^-$ T cells (0.26±0.07% versus 2.04±1.40%, $P<0.0001$ in non-treated mice and 1.90±0.82%, $P=0.001$ in the lower dose group) and also a small decrease of $CD4^+CD25^+Foxp3^+$ Treg cells (4.77±0.71% versus 6.69±1.35%, $P<0.0001$ in non-treated mice and 6.30±1.32%, $P=0.01$ in the lower dose group). To evaluate the balance between effector and regulatory cells, we calculated the ratio of $Foxp3^+/Foxp3^-$ T cells as an indicator. We found a significantly increased ratio, indicating a more pronounced regulatory phenotype of T cell response in mice immunized with a higher concentration of cBSA (15.86±6.99 versus 4.89±3.28, $P<0.001$ in non-treated mice and 4.50±3.48, $P=0.01$ in the lower dose group).

The splenic cytokine profile in mice immunized with a higher dose of cBSA indicates a Th2 immune response

To further evaluate the influence of the immunization on the T cell population, the balance of the Th1/Th2 immune response was studied by measuring cytokine levels in cell supernatants of ConA stimulated splenocytes. Mice immunized with a higher concentration of cBSA were found to have higher concentrations of IL-5 (108.8±86.8 pg/mL versus 22.1±23.2 pg/mL, $P<0.001$ in non-treated mice and 59.5±54.7 pg/mL, $P=0.08$ in the lower dose group) and IL-10 (239.6±125.7 pg/mL versus 119.0±101.9 pg/mL, $P<0.01$ in non-treated mice and 137.9±144.0 pg/mL, $P<0.05$ in the lower dose group) in the cell

supernatants, indicating activation of a Th2 immune response. No differences were detected for IL-4, IFN-γ, IL-2, IL-12, IL-1β, TNF-α and KC (data not shown). Furthermore, the immunization had no effect on the proliferation rate of ConA stimulated splenocytes (data not shown).

Decreased weight and plasma cholesterol levels in mice immunized with a higher dose of cBSA

There was a significant decrease in weight in mice immunized with a higher dose of cBSA compared to non-treated mice (Table 1). Interestingly, the same group was also found to have significantly lower plasma cholesterol levels (Table 1).

Discussion

In the present study we have demonstrated that immunization using the carrier cBSA inhibited lesion progression in ApoBec-1/LDL receptor deficient mice with advanced plaques. The immunization was also associated with a stabilization of the existing plaques in subvalvular lesions, shown by a reduced amount of macrophages and $CD3^+$ T cells as well as a higher content of collagen. This was also associated with an increased regulatory-to-effector T cell ratio as well as induction of an anti-inflammatory Th2 immune response, demonstrated with increased release of IL-5 and IL-10 in spleen cells and higher levels of apoB/IgG1 immune complexes in the circulation. Notably, immunization with both the low and high dose of the carrier protein resulted in induction of a robust antibody response against apoB, while only the high dose activated Treg cells and reduced progression of atherosclerosis. These observations point to a particularly important role of Treg activation and suggest that the beneficial effect of cBSA immunization is coupled to induction of a regulatory response against modified self antigens.

Novel anti-atherosclerotic therapies are most frequently tested in young animals, devoid of advanced plaques. Successful treatments in reducing atherosclerotic lesions have been reported using a number of approaches, but usually the mice were below 15 weeks of age when the treatment started (Ait-Oufella et al. 2006; Binder et al. 2003; Faria-Neto et al. 2006; Liu et al. 2006; van Puijvelde et al.

2006). Initiation of a treatment prior to development of severe disease certainly is a sound approach if the intention is to test prevention of disease initiation. However, autoimmune diseases in man usually require treatment to regress the disease. Previously we have reported a strategy that induced regression of atherosclerotic plaques in 25 weeks old ApoBec-1/LDL receptor deficient mice, using an IgG1 antibody directed towards an immunogenic apoB-100 peptide (Schiopu et al. 2007). The purpose of the present study was to find an active immunization approach resulting in regression or inhibition of the progression of advanced plaques. To meet this aim we decided to use the ApoBec-1/LDL receptor deficient mouse model which similar to humans expresses full-length apoB-100 (Hirano et al. 1996). Another advantage of these mice is that they express apoE, a molecule that have been implicated in lipid antigen presentation (van den Elzen et al. 2005). In a previous study we showed that a subcutaneous injection of alum in a hypercholesterolemic environment captured oxidized LDL antigens locally, resulting in activation of Treg cells and atheroprotection in young apoE deficient mice (Wigren et al. 2009). In line with this finding, we tested our hypothesis that in older mice there is a need of both alum and a carrier to potentiate the capture of antigens as well as more frequent injections, to induce an atheroprotective immune response. Thus, supposing that an aged and less responsive immune cell pool would require stronger and more frequent stimuli to exert an effect. Accordingly, we found a reduced plaque area in the descending aorta of mice immunized with the higher dose of cBSA and as

importantly, a more stable plaque phenotype, indicating that the findings are not only an effect of the alum included in the immunizations. A limitation of the present study, is lack of a group immunized with alum alone as part of a dose-response examination.

Evidence for a dysregulation of the T regulatory immune response in atherosclerosis development has been demonstrated (Mallat et al. 2007). Studies using Foxp3 deficient *Scurfy* mice and studies depleting the T-cell pool of $CD25^+$ cells show association with severe autoimmune inflammation and atherosclerosis (Ait-Oufella et al. 2006; Chang et al. 2005). Recently, van Es et al. reported that immunization of LDL receptor deficient mice with Foxp3-transfected dendritic cells, resulted in depletion of Treg cells and increased atherosclerosis (van Es et al. 2010). Taken together, these studies show a protective role of Treg cells in atherosclerosis development. Our result, that immunization with cBSA changed the balance of effector and Treg cells, resulting in a more prominent regulatory immune response and atheroprotection is well in line with previous findings.

It has been suggested that immunoglobulins contain regions or epitopes that stimulate Treg cells, i.e. Tregitopes (De Groot et al. 2008). Using computational epitope mapping De Groot et al. identified two clusters of major histocompatibility complex binding motifs in the Fc region of immunoglobulins

that could be presented to T cells. These Tregitopes were shown to activate and expand Treg cells, both natural and antigen-specific adaptive Tregs. Using the same method, six putative T cell epitope clusters have been identified in human serum albumin (data filed at CardioVax, Princeton, NJ). One of these was also found to be present in BSA. Thus, the more prominent regulatory immune response detected in our cBSA-immunized mice might be induced by the Tregitope in BSA resulting in reduced atherosclerosis. However, this assumption has to be further elucidated in future studies.

The presence of antibodies reactive to oxidation specific epitopes of LDL has been found in hyperlipidemic rabbits, mice and humans (Fredrikson et al. 2003; Palinski et al. 1989). Autoantibodies in human plasma have been shown to be associated with disease severity and risk for development of acute cardiovascular events (Nilsson and Kovanen 2004). However, we have recently presented an independent association between high levels of IgG antibodies to apoB-100 peptides and a low degree of carotid stenosis (Fredrikson et al. 2007), lower risk of myocardial infarction (Sjogren et al. 2008), and a lower risk for progression of coronary disease in diabetic patients (Fredrikson et al. 2009). The atheoprotective effect of IgG has also been shown in experimental studies using human IgG1 specific for a malondialdehyde-modified apoB-100 peptide (Schiopu et al. 2004; Schiopu et al. 2007). In accordance, we found in the present study increased levels of Th2-induced apoB/IgG1 immune complexes in

the circulation of cBSA immunized mice, indicating a capture of oxidized LDL antigens at the injection site and a subsequent presentation by APCs, resulting in atheroprotection. Additionally, the immunization resulted in increased levels of the Th2 polarized cytokines, IL-5 and IL- 10, in the spleen as well as lower plasma cholesterol levels. Collectively, these findings might explain the reduced development of atherosclerosis in immunized mice. In our previous study, where apoE deficient mice were immunized with alum alone, also a trend toward an increase in Th2-specific IgG1 recognizing an oxidized LDL epitope was detected (Wigren et al. 2009). In contrast, the alum treatment was found to reduce Con A-induced T cell proliferation, and had no effect on the splenic cytokine release. These discrepancies may be explained by the deviating design of the present study, such as the mouse model, the age of the mice and the immunization strategy used.

In conclusion, we have shown that immunization with cBSA, in a dose-dependent manner, results in inhibition of the progression of advanced lesions in ApoBec-1/LDL receptor deficient mice. The immunization was also found to result in enhanced Th2 and shift toward T regulatory immune responses, which may have a role in the detected atheroprotection.

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Conflict of interest

No conflicts of interest exist.

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Figure legends

Figure 1. Mice immunized with cBSA have more circulating apoB-100 containing immune complexes. Plasma from untreated (No treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA were analyzed for apoB specific IgG (A) and IgG1 (B) immune complexes. *** $P<0.001$ versus No treatment.

Figure 2. cBSA immunization reduces plaque development. Plaque areas in descending aortas of untreated (No treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA. Plaque areas in descending aortas were assessed by the *en face* Oil Red O staining and the percent stained area of total aortic area was determined by computerized image analysis. * $P<0.05$ versus No treatment and cBSA low.

Figure 3. Mice immunized with a higher dose of cBSA have more stable plaques in subvalvular lesions. The monocyte/macrophage (A) and the collagen (B) content were determined within subvalvular lesions from untreated (No treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA. The ratio (C) of collagen and monocyte/macrophage content in the plaques was calculated. ***$P<0.001$, ** $P<0.01$ and *$P<0.05$ versus No treatment and cBSA low.

Figure 4. Mice immunized with a higher dose cBSA have less $CD3^+$ T cells in subvalvular lesions. The $CD3^+$ T cell area of the total plaque area were determined within subvalvular lesions of untreated (No treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA. *** $P<0.001$ versus No treatment.

Figure 5. Mice immunized with a higher dose cBSA have increased regulatory-to-effector T cell ratios in the blood. Blood cells from untreated (No treatment) and mice immunized with 50 (cBSA low) or 100 μg (cBSA high) cBSA were stained with fluorochrome-conjugated antibodies recognizing CD4, CD25 or Foxp3 and analyzed with a CyAn ADP flow cytometer. The percentages given are (A) the $CD25^+FoxP3^-$ cells or (B) the $CD25^+FoxP3^+$ cells out of the $CD4^+$ T cell population, respectively. The ratio (C) of $Foxp3^+/Foxp3^-$ T cells in the blood was calculated. ** $P<0.01$ and *** $P<0.001$ versus No treatment and cBSA low.

Table 1. Mouse weight, plasma cholesterol and triglyceride levels.

Group	Weight (g)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
No treatment	24.2 ± 2.9	253 ± 26	54.6 ± 15.6
cBSA low	24.3 ± 1.5	239 ± 33	47.4 ± 11.1
cBSA high	22.3 ± 1.4*	232 ± 29*	46.7 ± 7.2

*P<0.05 versus No treatment. Values are mean ± SD. n = 10-21 per group.

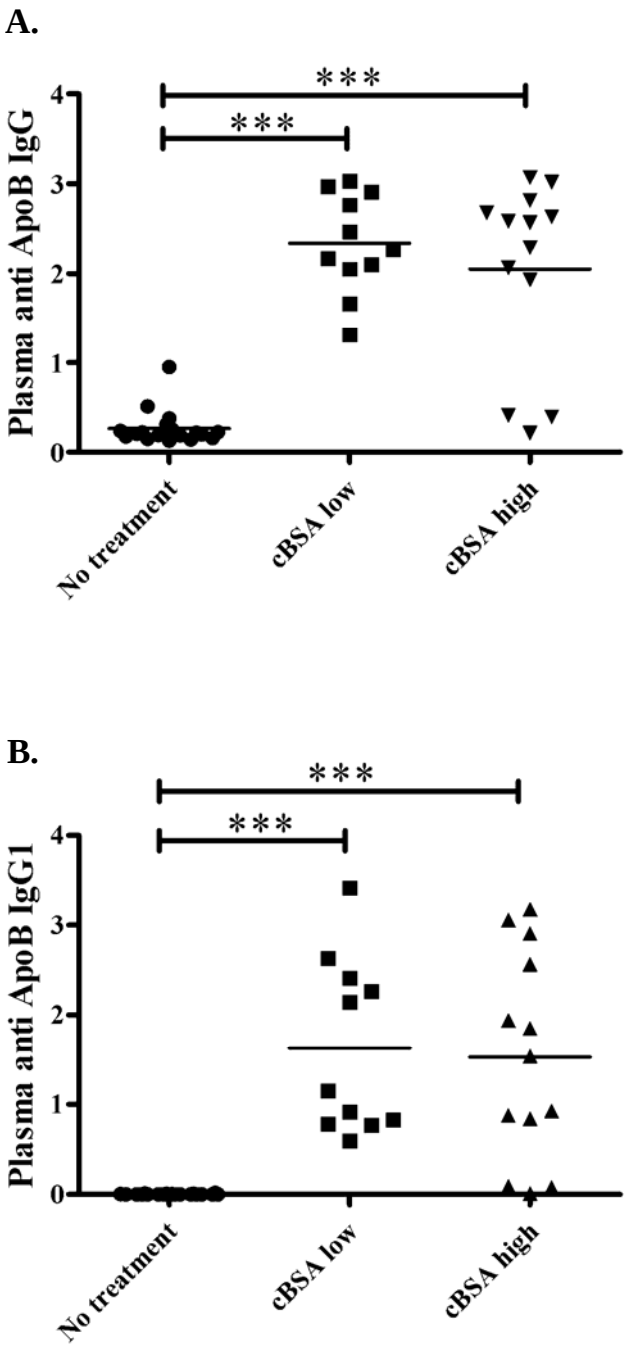
A.
***
***
Plasma anti ApoB IgG
4
3
2
1
0
No treatment
cBSA low
cBSA high
B.
***
***
Plasma anti ApoB IgG1
4
3
2
1
0
No treatment
cBSA low
cBSA high

Figure 1.

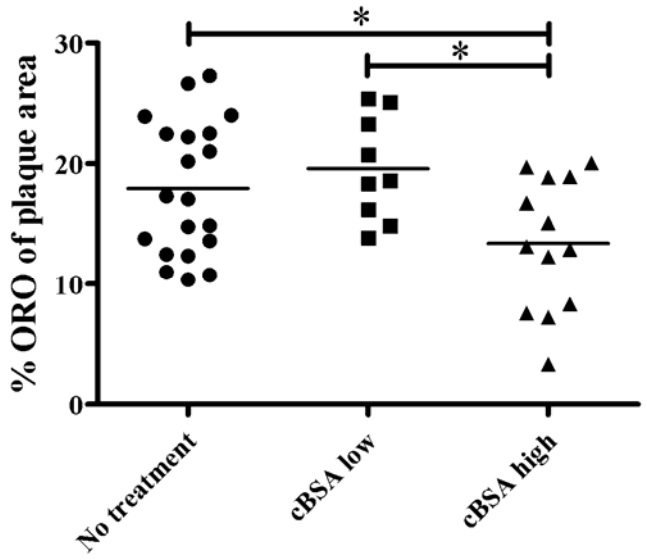

Figure 2.

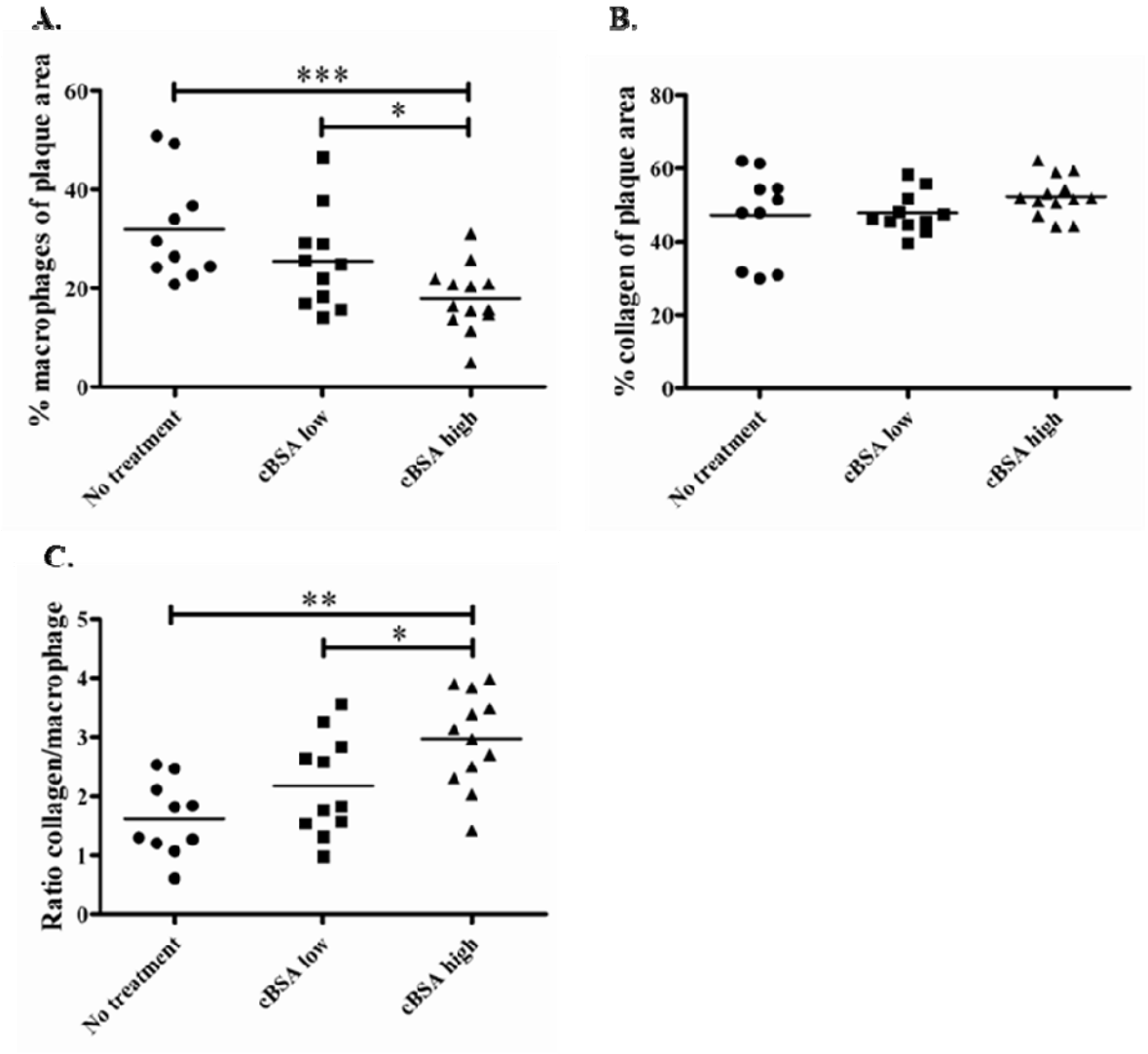
A.
% macrophages of plaque area
60
40
20
0
***
*
No treatment
cBSA low
cBSA high
B.
% collagen of plaque area
80
60
40
20
0
No treatment
cBSA low
cBSA high
C.
Ratio collagen/macrophage
5
4
3
2
1
0
**
*
No treatment
cBSA low
cBSA high

Figure 3.

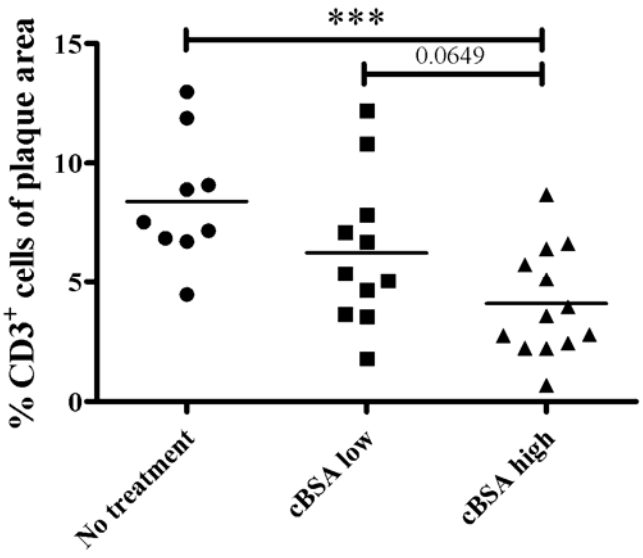
***
0.0649
% CD3+ cells of plaque area
15
10
5
0
No treatment
cBSA low
cBSA high

Figure 4.

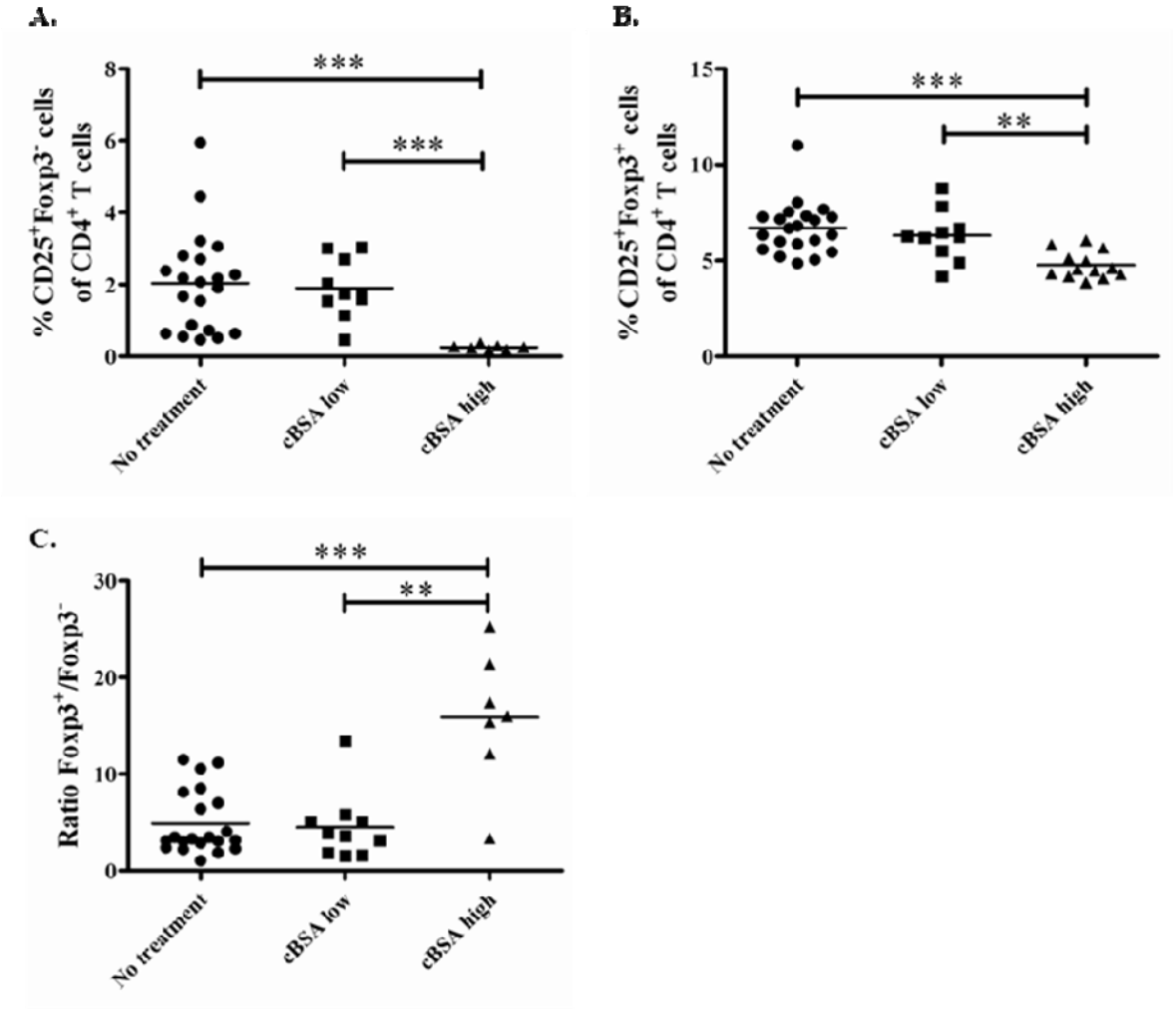
A.
% CD25+Foxp3- cells of CD4+ T cells
***
***
No treatment
cBSA low
cBSA high
B.
% CD25+Foxp3+ cells of CD4+ T cells
***
**
No treatment
cBSA low
cBSA high
C.
Ratio Foxp3+/Foxp3-
***
**
No treatment
cBSA low
cBSA high

Figure 5.